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Functional genomic screen for post-transcriptional 5' and 3' regulatory elements

Abstract:

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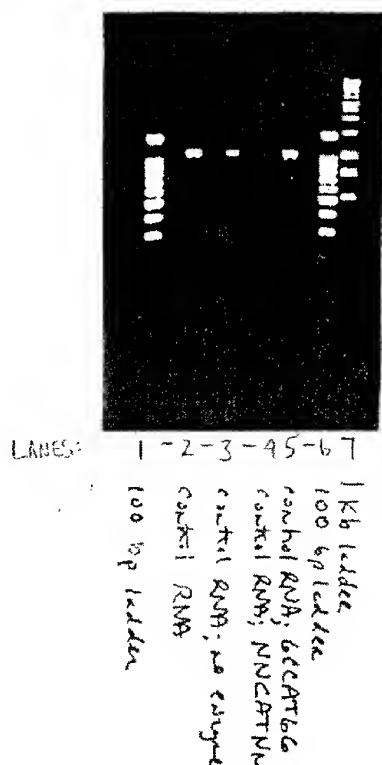
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(54) **Functional genomic screen for post-transcriptional 5' and 3' regulatory elements**

(57) The invention features cDNA libraries consisting essentially of cDNA sequences that correspond to different mRNA untranslated region (UTR) sequences separate from adjacent mRNA coding sequences. The invention also features methods for generating these libraries and for identifying a regulatory UTR sequence.

FIG. 1



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**Description**Field of the Invention

5 **[0001]** This invention relates to the field of nucleic acid regulatory elements that affect mRNA translation, export, and stability. More specifically, the invention relates to the screening of 5' and 3' untranslated RNA sequences, the identification of RNA regulatory elements within these sequences, and the identification of compounds that modulate the function of these RNA regulatory sequences.

10 Background

**[0002]** While transcriptional controls regulate gene expression by influencing the rate of mRNA production, post-transcriptional mechanisms can also regulate gene expression by modulating the amount of protein produced from an mRNA molecule. For example, gene expression can be regulated by altering mRNA translation efficiency (Izquierdo and Cuez, Mol. Cell Biol. 17: 5255-5268, 1997; Yang et al., J. Biol. Chem. 272: 15466-73, 1997), or by altering mRNA stability (Ross, Microbiol. Rev. 59: 423-50, 1995). Post-transcriptional control mechanisms appear to play an especially important role in the gene expression response to environmental factors, such as response to heat shock (Sierra et al., Mol. Biol. Rep. 19: 211-20, 1994), iron availability (Hentze et al., Proc. Natl. Acad. Sci. USA 93: 8175-82, 1996), oxygen availability (Levy et al., J. Biol. Chem. 271: 2746-53, 1996; McGary et al., J. Biol. Chem. 272: 8628-34, 1997), and growth factors (Amara et al., Nucleic Acids Res. 21: 4803-09, 1993).

**[0003]** Post-transcriptional regulatory elements may be present in the 5' and 3' mRNA untranslated regions (UTRs). At the 5' UTR, mRNA binding to ribosomes is generally the rate-limiting step in translation initiation (Mathews et al., In: Translational Control, pages 1-30, Eds: Hershey et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1996). At the 3' UTR, regulatory elements may modulate mRNA translation and degradation, as well as mRNA transport and subcellular localization (Jackson, Cell 74: 9-14, 1993). However, the nature of most UTR post-transcriptional elements remains poorly understood. A method for efficiently characterizing these mRNA regulatory sequences would advance the discovery of compounds that modulate expression of therapeutically important proteins via regulatory mRNA sites.

30 Summary of the Invention

**[0004]** We have discovered a method for constructing libraries that are specifically biased for RNA regulatory sites. In the first aspect, the invention features a cDNA library consisting essentially of at least 100 different cDNA sequences that correspond to different mRNA untranslated region (UTR) sequences isolated and separate from adjacent mRNA coding sequences. Preferably, the cDNA sequences are cloned into a vector system that can express the sequences, and such a vector is also a feature of this invention. This vector includes the following: a) a nucleotide sequence encoding an mRNA UTR sequence in operative linkage to a promoter, wherein the nucleotide sequence is derived from the cDNA library of the first aspect; b) a first reporter gene positioned for transcription upstream or downstream of the UTR-encoding nucleotide sequence; and c) a second, different reporter gene in operative linkage to a promoter but unassociated with the UTR-encoding nucleotide sequence. Preferably, the reporter genes encode a fluorescent protein or cell surface marker protein.

**[0005]** A second and related aspect of the invention features a cDNA library, wherein the library is constructed by steps that include the following: a) purifying poly(A)<sup>+</sup> RNA from total RNA; b) performing controlled, non-random enzymatic digestion of AUG sequences in the poly(A)<sup>+</sup> RNA; c) purifying the digested RNA to obtain the fragments containing the 5' end sequences; and d) synthesizing cDNA from the purified RNA obtained in step (c); wherein the library consists essentially of cDNA sequences corresponding to mRNA 5' untranslated region (UTR) sequences, isolated and separate from adjacent mRNA coding sequences. Preferably, the enzymatic digestion is carried out using RNase H.

**[0006]** In a third aspect, the invention features a cDNA library constructed by steps that include the following: a) purifying poly(A)<sup>+</sup> RNA from total RNA; b) synthesizing nucleic acid heteroduplexes from the poly(A)<sup>+</sup> RNA, using degenerate primers that hybridize preferentially to the region surrounding and including the initiation codon, where the heteroduplexes comprises the 5' end sequences of the RNA; c) purifying the heteroduplexes obtained in step (b) to obtain the fragments containing the 5' end sequences; and d) synthesizing cDNA from the purified heteroduplexes obtained in step (c); wherein the library consists essentially of cDNA sequences corresponding to mRNA 5' untranslated (UTR) sequences, isolated and separate from adjacent mRNA coding sequences.

**[0007]** In one embodiment of any of the above three aspects of the invention, the cDNA library consists essentially of cDNA sequences corresponding to mRNA untranslated region sequences, isolated in intact form.

**[0008]** In preferred embodiments of the second or third aspects of the invention, the 5' sequence purification is carried

out using a cap binding protein, for example, an eIF4E fusion protein or an antibody to the 5' cap, and the cDNA sequences are cloned into a vector system that can express the sequences. This vector includes the following: a) a nucleotide sequence encoding an mRNA UTR sequence in operative linkage to a promoter, wherein the nucleotide sequence is derived from the cDNA library of the second or third aspect; b) a first reporter gene positioned for transcription upstream or downstream of the UTR-encoding nucleotide sequence; and c) a second, different reporter gene in operative linkage to a promoter but unassociated with the UTR-encoding nucleotide sequence. Preferably, the reporter genes encode a fluorescent protein or cell surface marker protein.

**[0009]** A related fourth aspect of the invention is a cDNA library, wherein the library is constructed by steps that include the following: a) purifying poly(A)+ RNA from total RNA; b) performing random digestion on the poly(A)+ RNA; c) purifying the digested RNA to obtain poly(A) containing fragments; and d) synthesizing cDNA from the purified RNA obtained in step (c); wherein the library consists essentially of cDNA sequences corresponding to 3' UTR sequences, isolated and separate from adjacent mRNA coding sequences.

**[0010]** A cDNA library is also featured in the fifth aspect of the invention. This cDNA library is constructed by steps that include the following: a) purifying poly(A)+ RNA from total RNA; b) loading the poly(A)+ RNA with ribosomes; and c) performing reverse transcription on the loaded poly(A)+ RNA using an oligo(dT) primer and polymerase; wherein the library consists essentially of cDNA sequences corresponding to 3' UTR sequences, isolated and separate from adjacent mRNA coding sequences. Preferably, the cDNA sequences of the libraries of the fourth or fifth aspects are cloned into vector systems that can express the sequences, and such vectors are also a feature of this invention. These vectors include the following: a) a nucleotide sequence encoding an mRNA UTR sequence in operative linkage to a promoter, wherein the nucleotide sequence is derived from the cDNA library of the fourth or fifth aspect; b) a first reporter gene positioned for transcription upstream or downstream of the UTR-encoding nucleotide sequence; and c) a second, different reporter gene in operative linkage to a promoter but unassociated with the UTR-encoding nucleotide sequence. Preferably, the reporter genes encode a fluorescent protein or cell surface marker protein.

**[0011]** In one embodiment of the fourth or fifth aspect of the invention, the cDNA library consists essentially of cDNA sequences corresponding to 3' untranslated region sequences, isolated in intact form.

**[0012]** A sixth aspect of the invention provides a method of identifying a regulatory UTR sequence that includes the following steps: a) transfecting a plurality of host cells with a plurality of vectors of the present invention, wherein the host cells are transfected with different UTR sequences; b) sorting cells on the basis of the ratio between expression of the first reporter gene and the second reporter gene; c) identifying the cells of step a) that have skewed expression ratios as compared to the population of cells of step (a) as a whole, or as compared to cells transfected with a vector that encodes the first and second reporter gene, but lacks the corresponding UTR sequence; and d) sequencing the UTR expressed in the identified cells. Preferably, the gene expression is detected by emission of fluorescence and the cells are sorted by a fluorescence activated cell sorter.

**[0013]** The seventh and final aspect of the invention features a cell transfected with any of the vectors of the present invention.

**[0014]** By "different mRNA untranslated region (UTR) sequences" or "different UTR sequences" is meant sequences that differ from each other in that they are derived from different mRNA species. As used herein, mRNA UTR sequences that are products of alternated splicing are considered to be different mRNA UTR sequences.

**[0015]** By "controlled, non-random enzymatic digestion of AUG sequences" is meant preferentially digesting mRNA at the site of AUG sequences, for example, using RNase H and a mixture of degenerate AUG-complementary oligonucleotide 7-mers, under conditions that require hybridization of more than 5 consecutive base pairs for RNase substrate recognition. To preferentially digest the initiation-AUG sequences in an mRNA population, the 7-mers in the AUG-complementary oligonucleotide mixture used have frequencies of A, C, G, and T at each position that are complementary to the frequencies of A, C, G, and U occurring in all known vertebrate mRNA sequences between the -3 and +4 position (where +1 is the first nucleotide of the coding sequence) (see, e.g., Table 1).

**[0016]** By "UTR sequences isolated and separate from adjacent mRNA coding sequences" is meant the following: 1) 5' UTR sequences that begin at the 5' end of a transcribed mRNA and extend up to, but do not include, the translation AUG initiation site; and 2) 3' UTR sequences that begin at the mRNA nucleic acid in the position 3' adjacent to the translation termination site and extend to the poly(A) tail of the transcribed mRNA. Preferably, the UTR sequences are isolated in intact form.

**[0017]** By "random digestion" of poly(A)+ RNA is meant RNase digestion using, for example, RNase H and random primers to digest the RNA into smaller fragments at random sites.

**[0018]** By "loading poly(A)+ RNA with ribosomes" is meant contacting the RNA population with ribosomes, for example, in a rabbit reticulocyte lysate, to allow for loading of the ribosomes onto the RNA. To maximize ribosome loading, a chemical that prevents ribosome runoff, for example, cycloheximide, can be included.

**[0019]** By a "plurality" is meant more than one.

**[0020]** By "skewed expression ratios" is meant a change in the ratio of expression of a first reporter gene that is associated with a specific UTR to expression of a non-UTR associated second reporter gene, as compared to the ratio

of expression of the first reporter gene that is not associated with the same UTR compared to expression of the non-UTR associated second reporter gene.

**[0021]** The screening assay and the 5' and 3' mRNA untranslated region (UTR) biased cDNA libraries of the present invention have a number of advantages. The biased UTR libraries provide a collection of UTR sequences that are isolated and separated from any adjacent coding sequences. Thus, screening these libraries allows opportunity to screen essentially complete UTR sequences without interference from coding sequences. In addition, the quantity of sequences screened and the specificity of output can be modulated by controlling conditions that regulate the number of different plasmids that enter each cell. In most circumstances, the ideal number of plasmids per cell would be limited to one, thereby reducing signal dilution and the occurrence of false negative results.

**[0022]** Other features and advantages of the invention will be apparent from the detailed description thereof and from the claims.

#### Description of the Figures

**[0023]** Fig. 1 demonstrates RNase H digestion of a control RNA sequence using specific or partially degenerate oligodeoxynucleotide 7-mers, under conditions that allow hydrolysis only if 6 or more consecutive base pairs are hybridized (compare lanes 4 and 5).

**[0024]** Fig. 2 demonstrates RNase H digestion of a control sequence using two different sequence specific oligodeoxynucleotides, under conditions that allow hydrolysis only if 7 consecutive base pairs are hybridized (see lane 3).

**[0025]** Fig. 3 shows RNase H digestion of poly(A)+RNA using a partially degenerate oligonucleotide 7-mer, under conditions that allow hydrolysis only if 7 consecutive base pairs are hybridized. The number of hydrolysis sites can be limited, even after extended incubation (compare lanes 6 and 7).

**[0026]** Fig. 4 illustrates limited reverse transcription of 3' UTR sequences.

#### Detailed Description

**[0027]** The practice of the present invention employs conventional techniques in biochemistry, molecular biology, microbiology, and related fields that are known to those skilled in the art. These techniques are fully explained in the literature (see, e.g., Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (1982); Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press (1989); and Ausubel, et al., *Current Protocols in Molecular Biology*, John Wiley & Sons (1987-1996 ed.)

#### Construction of UTR Libraries

**[0028]** Poly(A)+ RNA is isolated from total cellular RNA, according to standard protocol (Aviv and Leder, *Proc. Natl. Acad. Sci. USA* 69: 1408-12, 1972).

**[0029]** To construct 5' UTR biased libraries, poly(A)+ RNA is subjected to controlled, non-random enzymatic digestion followed by size selection. The enzymatic digestion of the poly(A)+ RNA is carried out, for example, using *E. coli* RNase H in the presence of a 7-mer oligodeoxynucleotide mixture, wherein the sequences of the oligodeoxynucleotides have A, C, G, and T at frequencies of occurrence that are complementary to the frequencies of occurrence of A, C, G, and U in all known vertebrate mRNA sequences between the -3 and +4 positions of the mRNA (where position 1 of the oligodeoxynucleotide is complementary to position +4 on the mRNA and position 7 is complementary to position -3 on the mRNA; see Table 1).

Table 1.

Designing Degenerate Oligonucleotides for the Isolation of 5' UTRs								
		+4	+3	+2	+1	-1	-2	-3
	7-mer oligodeoxynucleotide position	1	2	3	4	5	6	7
7-mer oligo-	A	15	0	100	0	9	11	1
deoxynucleotide	C	46	100	0	0	21	13	36
frequency (%)	G	16	0	0	0	55	49	2
	T	23	0	0	100	15	27	61

**[0030]** Given that *E. coli* RNase H requires hybridization of four consecutive base pairs in order to recognize a DNA/

RNA duplex region as a substrate (Donis-Keller, *Nucleic Acids Res.* 7: 179-192, 1979), the controlled RNase H digestion using the above-described oligodeoxynucleotides will primarily hydrolyze the initiation codon, but because of the degeneracy of the oligodeoxynucleotide mixture, and the minimum consecutive number of base pairs required under physiological conditions, RNase H can also hydrolyze the RNA at many other locations, including regions in the 5' UTR. To further restrict the digestion to the initiation codon, conditions can be modified such that RNase H recognition requires hybridization of more than five base pairs (see Example 1). The AUG sequence is rare within the 5' UTR sequences (Kozak, *Nucleic Acids Res.* 15: 8125-48, 1987). Therefore, this RNase H digestion will preferentially result in intact, full-length 5' UTR sequences that are separated from the adjoining coding sequences.

**[0031]** To enrich the population of 5' UTR-containing fragments within the mRNA sample, fragments of up to 1000 nucleotides are selected using denaturing agarose gels. The 5' UTRs of most vertebrate mRNAs fall within the size range of 20-100 nucleotides (Kozak, *supra*). Subsequent to size selection, the mRNA sample is subjected to affinity purification using a recombinant eIF4E fusion protein that interacts with the mRNA 5' cap structure (Sonenberg and Gingras, *Curr. Opin. Cell Biol.* 10: 268-75, 1998).

**[0032]** An alternative strategy for isolating 5' UTRs from purified poly(A)+ RNA is to reverse transcribe the poly(A)+ RNA using a degenerate (*i.e.*, mixed-sequence) primer that hybridizes preferentially to the region surrounding and including the initiation codon (the 3' border of the 5' UTR).

**[0033]** The consensus sequence surrounding the initiation codon of vertebrate mRNAs is GCC (G/A)CC AUG G (SEQ ID NO: 1), where the underlined sequence is the initiation codon, and the nucleotides in parentheses are found with nearly equal frequency at that position.

**[0034]** A degenerate primer complementary to this consensus sequence can be designed that takes into account all the variations in frequency of the nucleotides at each position, so that the primer mixture has a high probability of hybridizing specifically to the initiation codon region. Table 2, below, shows that primers can be designed, based on the known sequences of hundreds of vertebrate mRNAs.

Table 2.

Designing Degenerate Primers for the Isolation of 5' UTRS											
mRNA 3'	+ 4	+ 3	+ 2	+ 1	-1	-2	-3	-4	-5	-6	5'
Primer 5'	1	2	3	4	5	6	7	8	9	10	3'
%A	10	0	100	0	10	10	5	10	20	20	
%C	50	100	0	0	20	15	30	15	20	45	
% G	20	0	0	0	55	50	5	50	40	20	
% T	20	0	0	100	15	25	60	25	20	15	
Data based on Kozak, <i>Nucleic Acids Res.</i> 15, 8125-8148, 1987 and Kozak, <i>Gene</i> 234:187-208, 1999.											

**[0035]** Referring to Table 2, the mixed-sequence primer reading 5' to 3' is complementary to the mRNA sequence surrounding the initiation codon. The numbering across the top from +4 through -6 corresponds to the numbering for the mRNA sequence, where position +1 is the first nucleotide of the initiation codon, and all the negative numbers refer to nucleotides in the 5' UTR. The percentages refer to the frequency of occurrence of a given nucleotide at a given position. Therefore, the primer would be synthesized such that, for example, at position 5, A occurs 10% of the time, C occurs 20% of the time, G occurs 55% of the time, and T occurs 15% of the time. Note that positions 2, 3, and 4 are invariant as they are complementary to the initiation codon, AUG. It is expected that a degenerate primer of the above composition would hybridize preferentially to the region of the mRNA surrounding and including the initiation codon.

**[0036]** Following RT-PCR to generate a minus strand cDNA hybridized to mRNA, the heteroduplex can be isolated by affinity purification of the complex. The mRNA/cDNA hybrids are incubated with either a monoclonal antibody to the 5' cap or a cap-binding protein, for example, an eIF4E protein attached to a solid matrix, washed and eluted to enrich for RNAs containing the full 5' UTR. Following elution of the complex, the RNA is digested with RNase H and terminal transferase is used to label the 3' end of the cDNA with poly d(T). Poly d(A) is then used to prime the second strand synthesis of the cDNA. The 5' UTR enriched library is then cloned 5' to the reporter gene.

**[0037]** To construct the 3' UTR biased libraries, poly(A)+ RNA is digested, for example, using random primers and *E. coli* RNase H, followed by selection of poly(A)-containing fragments using oligo(dT)-linked resin. The isolated poly(A)-containing fragments are incubated with reverse transcriptase using oligo(dT) primers. Alternatively, to retrieve mRNA that is exclusively 3' UTR, isolated RNA is allowed to associate with ribosomes, for example, in lysates from rabbit reticulocytes. Under conditions in which ribosome run-off is inhibited by cycloheximide, reverse transcription is

performed in the presence of oligo(dT) and a low efficiency polymerase (see Example 2).

**[0038]** The purified 5' and 3' UTR RNA fragments are subjected to 5' RACE (Rapid Amplification of cDNA Ends) to obtain double-stranded cDNA (Frohman, In: PCR Protocols: A Guide to Methods and Applications, pages 28-38, Eds: Innis et al., Academic Press, London). The 5' or 3' UTR cDNAs are then ligated into an expression vector of choice, for example, a retroviral vector. The 5' and 3' UTR sequences are positioned upstream or downstream, respectively, of a reporter gene's coding sequence.

#### Screening Assay

**[0039]** The expression vectors used for transfection of host cells each encode one UTR, in operative linkage to a promoter, linked to its UTR-associated first reporter gene. The vector also includes a second, different reporter gene that is operably linked to a promoter, but is not associated with the UTR. Expression of this UTR-independent second reporter gene is not regulated by any UTR effect. Thus, expression of this second reporter gene controls for differences in expression that result from variations in plasmid number or transcriptional efficiency. In addition, conditions can be varied to reduce the number of different vectors, and, thus, the number of UTRs, that are introduced into each cell. To carry out host cell transfection, conditions are adopted to limit transfection, preferably, to less than 5 plasmids per cell, most preferably, to one plasmid per cell. Usually, it is preferable to identify conditions that allow nearly clonal delivery of the vectors to the cells. For retroviral transduction methods, cells are infected at a multiplicity of infection (MOI) such that each cell is infected with approximately one virus. The MOI can be determined empirically for each cell line and construct. Alternatively, plasmids can be delivered to cells via protoplast fusion (Tan and Frankel, Proc. Natl. Acad. Sci. 95:4247-52, 1998). For this method, *E. coli* are transformed with plasmid libraries, the bacteria cell walls are removed and the resulting protoplasts are fused to mammalian cells with polyethylene glycol. By adjusting the ratio of protoplasts to mammalian cells, plasmid delivery is reported to be nearly clonal, with individual cells containing 1000 copies of a single plasmid.

**[0040]** The choice of cell type to be used will depend on several factors, for example, the biological system of interest and the ease of foreign DNA transfection. Thus, if the biological system of interest is breast cancer-related genes, a breast cancer cell line may be used. In addition, given that retroviral transduction may be the only efficient means of transfection in some cell lines, use of these cells will not be preferred if another means of transfection is desired.

**[0041]** Expression of the UTR-associated reporter gene will be compared to expression of the non-UTR associated second reporter gene. Any discrepancies in this ratio of expression could reflect UTR-mediated changes in mRNA translation, export, or stability. Many potential schemes for detecting expression, and identifying expression-altering UTRs are available. Particularly well-suited systems are those that produce a colored or otherwise detectable product as determined by gel electrophoresis, detection of fluorescence, chemiluminescence, or antibody binding. For example, cells that express such UTRs can be identified and isolated using a fluorescence activated cell sorter (FACS) and green fluorescent protein (GFP) as a reporter gene (Bierhuizen et al., Biochem. Biophys. Res. Commun. 234: 371-375, 1997; Grignani et al., Cancer Res. 58: 14-19, 1998; de Martin et al., Gene Ther. 4: 493-495, 1997; Foster et al., J. Virol. Methods 75: 151-60, 1998). Such a system is advantageous for high throughput screening. Other systems that can be used to track gene expression include detecting *E. coli* lacZ-encoded  $\beta$ -galactosidase activity coupled with a fluorogenic substrate (Fiering et al., Cytometry 12: 291-301, 1991) and detecting the expression of foreign cell-surface antigens by means of fluorescently-labeled antibodies (Planelles et al., Gene Ther. 2: 369-76, 1995).

**[0042]** In the case of detection by fluorescence, the emission spectra of the fluorophores used to track expression of the UTR-associated first reporter genes and non-UTR associated second reporter genes must be sufficiently different so that, for example, the FACS instrument can perform two-color analysis and sort cells on the basis of the correlation between expression of the two reporter genes. The transfected cell population will consist of four different expression patterns as follows: 1) cells that are negative for both gene markers, indicating transfection failure; 2) cells with a ratiometric relationship between expression of the UTR-linked gene and the control gene, indicating that the UTR has no effect on gene expression; 3) cells with disproportionately higher expression of the UTR-linked gene, indicating that the UTR enhances translation efficiency or mRNA stability; and 4) cells with disproportionately lower UTR-linked gene expression, indicating that the UTR reduces translation efficiency or mRNA stability.

**[0043]** Following FACS sorting, cells with skewed fluorescence signals can be collected for further analysis. The sequence of the expression-altering UTR can be determined using, for example, PCR with vector primers, or plasmid rescue. One fluorescent color readout is dependent upon levels of expression of the non UTR-linked second reporter gene and the other color is dependent upon the levels of expression of the UTR-linked gene. The FACS instrument is capable of determining the levels of expression of both colors simultaneously and plots the two levels for each individual cell versus each other. It is expected that most UTRs will not affect gene expression and therefore, a majority of the transfected cells should express a consistently proportional level of both gene products. This population of cells will occupy a characteristic region of the two color plot. Cells that fall outside of this region will be automatically sorted into one of two tubes with UTR-linked genes that proportionally up-regulate gene expression in one tube and UTR-linked

genes that down-regulate gene expression in the other.

**[0044]** A similar strategy can be used to screen and identify compounds that affect the function of the 5' and 3' UTR regulatory elements. Compounds that modulate the UTR effect on gene expression would skew the expression of the UTR-linked gene as compared to gene expression in the absence of the compound.

*Example 1: Selective RNase H Digestion of mRNA*

**[0045]** Conditions for digestion can be adopted that prevent RNase H hydrolysis unless mRNA hybridization to the oligodeoxynucleotide probe encompasses more than 5 or 6 consecutive nucleotides. This was demonstrated in an experiment in which a 7-mer oligodeoxynucleotide was designed to hybridize to a control mRNA species at multiple locations, but to form no more than five consecutive DNA/RNA base pairs at any one of these locations. No hydrolysis occurred using this oligodeoxynucleotide, but it did occur using a partially degenerate oligodeoxynucleotide, NNCATNN (where N is an equimolar mixture of A, C, G, and T) which allowed hybridization of 6 or 7 consecutive base pairs (see Fig. 1). Following denaturation of 0.2 µg control RNA (Promega luciferase control sequence) and 70 pmol oligodeoxynucleotide in 10 mM Tris HCl, pH 8.0, 50 mM NaCl, at 70° C for 10 minutes, samples were submerged in ice. RNase H, MgCl<sub>2</sub>, and DTT were added to final concentrations of 0.4 units, 5 mM, and 1 mM, respectively. Samples were incubated at 20° C for 60 minutes. The reactions were terminated by the addition of EDTA to a final concentration of 25 mM, and digestion products were separated and visualized on a 1% TBE non-denaturing agarose gel stained with ethidium bromide.

**[0046]** Conditions for RNase digestion can also be controlled such that a sequencespecific oligodeoxynucleotide 7-mer will mediate RNase H-catalyzed hydrolysis of RNA only at the single site where seven consecutive DNA/RNA base pairs can form (see Fig. 2). These conditions included denaturing 0.2 µg control RNA (Promega luciferase control sequence) and 250 nmol oligodeoxynucleotide in 10 mM Tris HCl, pH 8.0, 50 mM NaCl, at 70 C for 10 minutes before submerging the samples in ice. Following the addition of RNase H, MgCl<sub>2</sub>, and DTT, as described above, and incubation at 20° C for 60 minutes, the digestion was terminated with the addition of EDTA to a final concentration of 25 mM. Digestion products were separated and visualized on a 6% polyacrylamide gel stained with ethidium bromide.

**[0047]** A population of poly(A)+ RNA can be substituted for a control mRNA, and the poly(A)+ RNA can be partially hydrolyzed with a degenerate oligodeoxynucleotide, as shown in Fig. 3. Thus, under conditions that prevent formation of fewer than seven consecutive DNA/RNA base pairs for hydrolysis by RNase H, a partially degenerate oligodeoxynucleotide can be used in the reaction with poly(A)+ RNA, and the number of hydrolysis sites can still be limited, even after an extended incubation period.

*Example 2: Use of Ribosomes to Construct Full Length 3' UTRs*

**[0048]** Using reverse transcription and an oligo(dT) primer, a full length 3' UTR sequence can be copied to cDNA. Reverse transcription begins with the poly(A) region and proceeds upstream towards the 5' end of the 3' UTR. To terminate transcription at the coding sequence termination site, the mRNA is fully loaded with actively translating ribosomes which cause steric hindrance of the transcriptase. Given that ribosomes do not bind mRNA downstream of the termination codon, the reverse transcriptase proceeds unhindered to copy the entire 3' UTR sequence, but the activity of the reverse transcriptase is then terminated, effectively separating the full length 3' UTR from any upstream coding sequence (see Fig. 4).

Other Embodiments

**[0049]** All publications mentioned herein are hereby incorporated by reference.

**Claims**

1. A cDNA library consisting essentially of at least 100 different cDNA sequences that correspond to different mRNA untranslated region (UTR) sequences isolated and separate from adjacent mRNA coding sequences.
2. A cDNA library, wherein said library is constructed by steps comprising
  - a) purifying poly(A)+ RNA from total RNA;
  - b) performing controlled, non-random enzymatic digestion of AUG sequences in the poly(A)+ RNA;
  - c) purifying said digested RNA to obtain the fragments containing the 5' end sequences; and
  - d) synthesizing cDNA from the purified RNA obtained in step (c);



wherein said library consists essentially of cDNA sequences corresponding to mRNA 5' untranslated region (UTR) sequences, isolated and separate from adjacent mRNA coding sequences.

3. The cDNA library of claim 2, wherein said enzymatic digestion is carried out using RNase H.

4. A cDNA library, wherein said library is constructed by steps comprising

- a) purifying poly(A)+ RNA from total RNA;
- b) synthesizing nucleic acid heteroduplexes from said poly(A)+ RNA using degenerate primers that hybridize to the region surrounding and including the initiation codon, said heteroduplexes comprising the 5' end sequences of said RNA;
- c) purifying the heteroduplexes obtained in step (b) to obtain the fragments containing the 5' end sequences; and
- d) synthesizing cDNA from the purified heteroduplexes obtained in step (c);

wherein said library consists essentially of cDNA sequences corresponding to mRNA 5' untranslated (UTR) sequences, isolated and separate from adjacent mRNA coding sequences.

5. The cDNA library of claim 2 or 4, wherein said 5' sequence purification is carried out using a cap binding protein.

6. A cDNA library, wherein said library is constructed by the steps comprising

- a) purifying poly(A)+ RNA from total RNA;
- b) performing random digestion on the poly(A)+ RNA;
- c) purifying said digested RNA to obtain poly(A) containing fragments; and
- d) synthesizing cDNA from the purified RNA obtained in step (c);

wherein said library consists essentially of cDNA sequences corresponding to 3' UTR sequences, isolated and separate from adjacent mRNA coding sequences.

7. A cDNA library, wherein said library is constructed by steps comprising

- a) purifying poly(A)+ RNA from total RNA;
- b) loading said poly(A)+ RNA with ribosomes; and
- c) performing reverse transcription on said loaded poly(A)+ RNA using an oligo(dT) primer and polymerase;

wherein said library consists essentially of cDNA sequences corresponding to 3' UTR sequences, isolated and separate from adjacent mRNA coding sequences.

8. The cDNA library of claim 1,2,4 wherein said cDNA sequences are cloned into a vector system that can express said sequences.

9. The cDNA library of claim 1, 2, 4, 6 or 7 wherein said UTR sequences are isolated in intact form.

10. A vector comprising

- a) a nucleotide sequence encoding an mRNA UTR sequence in operative linkage to a promoter, wherein said nucleotide sequence is derived from the cDNA library of claim 1, 2, 4, 6 or 7;
- b) a first reporter gene positioned for transcription upstream or downstream of said UTR-encoding nucleotide sequence; and
- c) a second, different reporter gene in operative linkage to a promoter but unassociated with said UTR-encoding nucleotide sequence.

11. The vector of claim 10 wherein said reporter genes encode a fluorescent protein or cell surface marker protein.

12. A method of identifying a regulatory UTR sequence, said method comprising

- a) transfecting a plurality of host cells with a plurality of vectors of claim 11, wherein said host cells are trans-

fectected with different UTR sequences;

b) sorting cells on the basis of the ratio between expression of the first reporter gene and the second reporter gene;

5 c) identifying the cells of step (a) that have skewed expression ratios as compared to the population of cells of step (a) as a whole, or as compared to cells transfected with a vector that encodes the first and second reporter gene, but lacks the corresponding UTR sequence; and

d) sequencing the UTR expressed in said identified cells.

10 13. The method of claim 12 wherein said gene expression is detected by emission of fluorescence.

14. The method of claim 13 wherein said cells are sorted by a fluorescence activated cell sorter.

15. A cell transfected with the vector of claim 10.

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FIG. 1

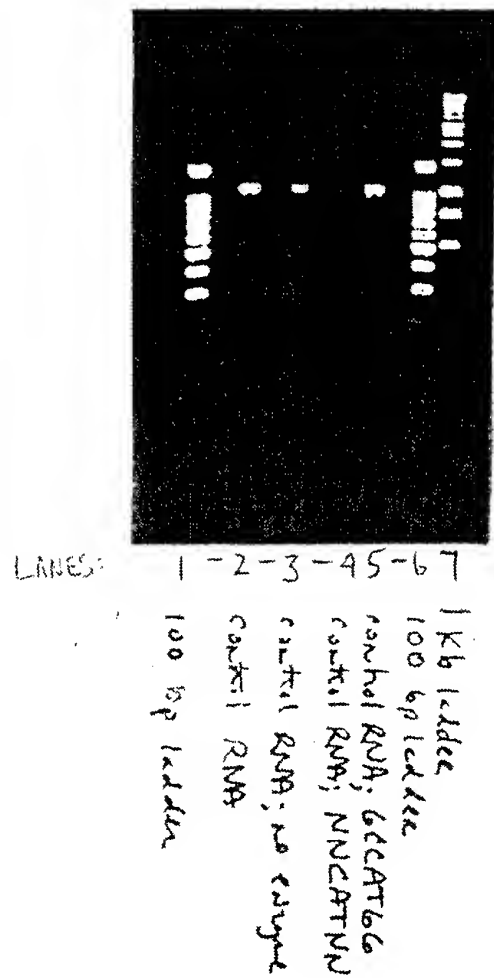
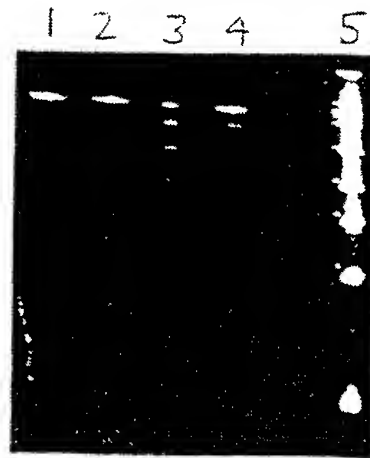


FIG. 2

Lanes:



RNA ladder

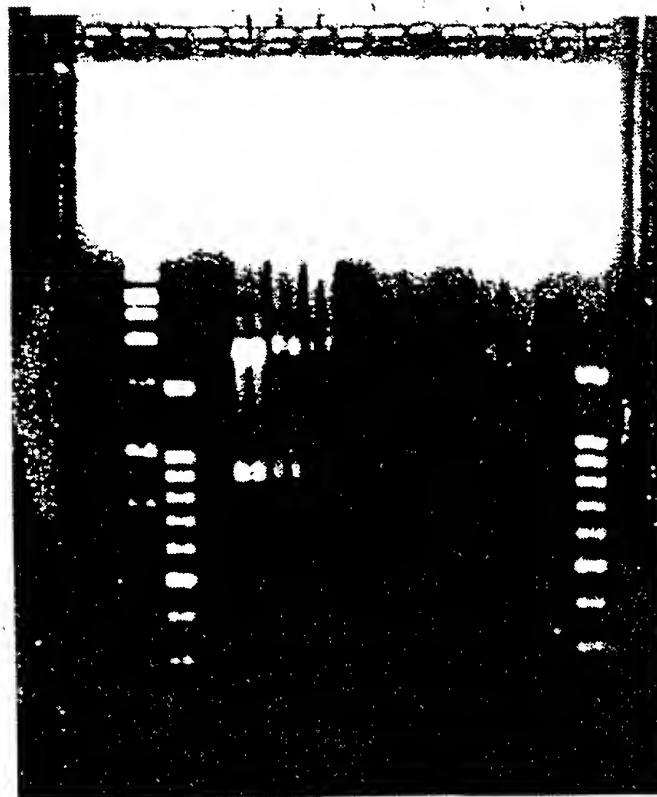
control RNA; 6CATT6C + GCATT6C

control RNA; 6CATT6C

control RNA; no enzyme

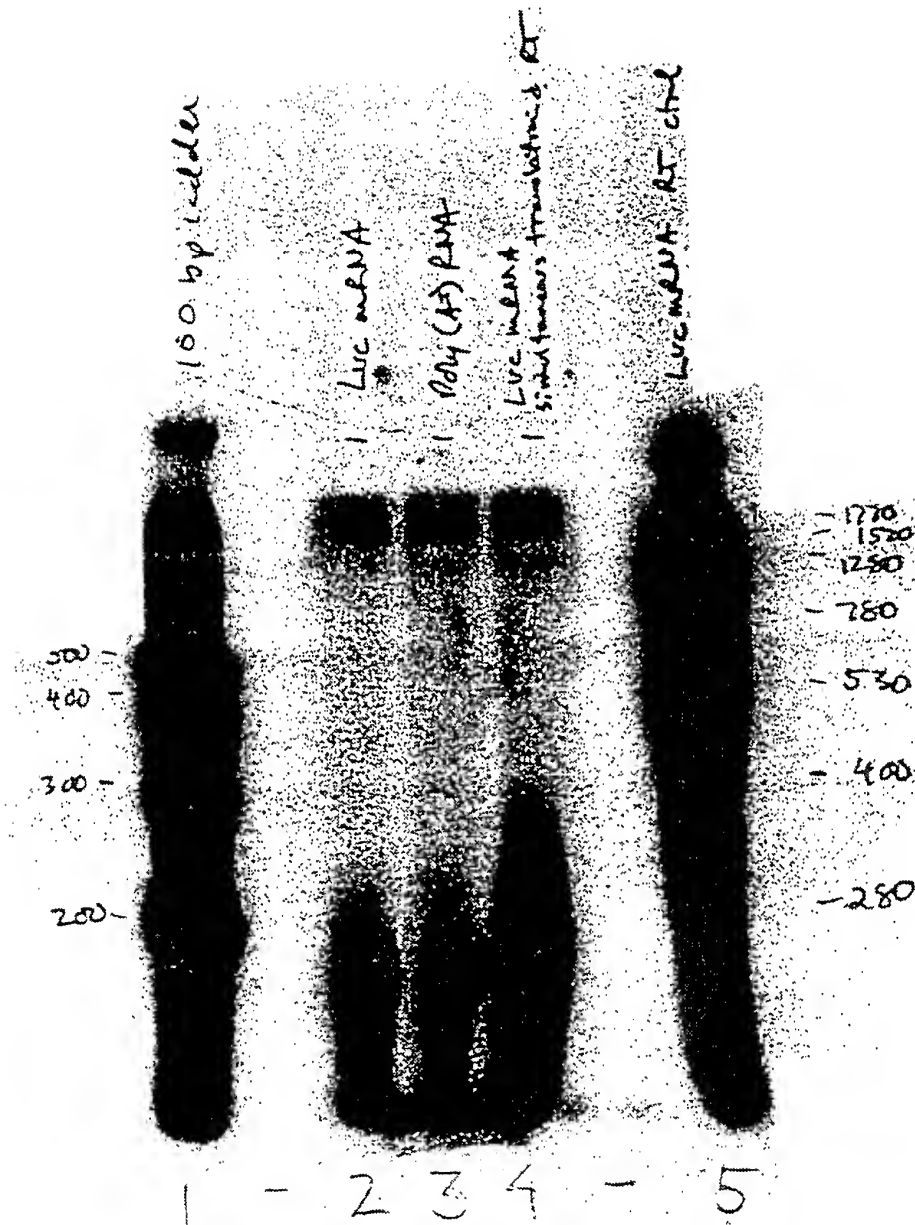
control RNA

FIG. 3



Lanes: 1 2-3 4 5 6 7 8 9 10  
 100 bp ladder  
 poly(A)+RNA; 6CCATG6  
 30' or 40'  
 poly(A)+RNA; NCATNN  
 30' or 40'  
 poly(A)+RNA; no enzyme  
 poly(A)+ADP  
 total RNA stock  
 100 bp ladder  
 1 kb ladder

FIG. 4





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## EUROPEAN SEARCH REPORT

Application Number  
EP 00 11 5854

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	WO 98 55502 A (SMITHKLINE BEECHAM CORP ; BERGSMAN DERK J (US); MOONEY JEFFREY L (US) 10 December 1998 (1998-12-10) * page 4, line 25-29 * * page 6, line 17-30 * ---	1,4,5,8,9	C12N15/10 C12N15/67
X	SUZUKI Y ET AL: "CONSTRUCTION AND CHARACTERIZATION OF A FULL LENGTH-ENRICHED AND A 5'-END-ENRICHED CDNA LIBRARY" GENE: AN INTERNATIONAL JOURNAL ON GENES AND GENOMES, ELSEVIER SCIENCE PUBLISHERS, BARKING, GB, vol. 200, 1997, pages 149-156, XP002932531 ISSN: 0378-1119 * the whole document * ---	1,4,8,9	
X	US 6 083 727 A (TAN RUOYING ET AL) 4 July 2000 (2000-07-04) * column 1, line 60 - column 2, line 8; claims 11-14; figure 1 * ---	1,8,9	
X	WO 94 23041 A (RIBOGENE INC) 13 October 1994 (1994-10-13) * figure 6; examples 19-25 * -----	10-15	TECHNICAL FIELDS SEARCHED (Int.Cl.7) C12N
<del>The present search report has been drawn up for all claims</del>			
Place of search BERLIN		Date of completion of the search 12 July 2001	Examiner ALCONADA RODRIG., A
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ..... 8 : member of the same patent family, corresponding document	

EPO FORM 1505 (03.82) (PatCat)



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### CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

- ☐ Only part of the claims have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid, namely claim(s):
- ☐ No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.

### LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

see sheet B

- ☐ All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.
- ☐ As all searchable claims could be searched without effort justifying an additional fee, the Search Division did not invite payment of any additional fee.
- ☐ Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid, namely claims:
- ☒ None of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims, namely claims:

1,8-15 (partially) and 2,3,4,5 (complete)





European Patent  
Office

LACK OF UNITY OF INVENTION  
SHEET B

Application Number  
EP 00 11 5854

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

1. Claims: 1,8-15 (partially) and 2,3,4,5 (complete)

A cDNA library consisting essentially of mRNA 5' untranslated regions and which is constructed by (i) purifying poly(A)+ RNA from total RNA; performing controlled non-random digestion of AUG sequences in the poly(A)+ RNA; purifying said digested RNA to obtain fragments containing the 5' end sequences and synthesizing cDNA from the purified RNA or (ii) purifying poly(A)+ RNA from total RNA, synthesizing nucleic acid heteroduplexes from said poly(A)+ RNA using degenerate primers, purifying said heteroduplexes; and synthesizing cDNA from the purified heteroduplexes; said cDNA library cloned in a vector system; said cDNA library wherein said UTR sequences are isolated in intact form; a bicistronic vector comprising an mRNA UTR in operative linkage to a promoter, wherein said nucleotide sequence is derived from the cDNA library, a first gene associated to said UTR and a second gene unassociated to said UTR; a method to identify a regulatory UTR sequence by using the bicistronic vector.

2. Claims: 1,8-15 (partially) and 6 (complete)

As invention 1 but relating to a cDNA library which essentially consists of mRNA 3' untranslated regions, said library being obtained by purifying poly(A)+ RNA from total RNA, performing random digestion on the poly(A)+ RNA; purifying said digested RNA to obtain poly(A) containing fragments and synthesizing cDNA from the purified RNA previously obtained.

3. Claims: 1,8-15 (partially) and 7 (complete)

As invention 2 but relating to a cDNA library obtained by purifying poly(A)+ RNA from total RNA, loading said poly(A)+ RNA on ribosomes; performing reverse transcription on said poly(A)+ RNA using an oligo(dT) primer.

**ANNEX TO THE EUROPEAN SEARCH REPORT  
ON EUROPEAN PATENT APPLICATION NO.**

EP 00 11 5854

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report.  
The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

12-07-2001

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82